# ANDROGENETIC DEVELOPMENT OF THE EGG OF RANA PIPIENS[1]

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## Introduction

The aim of the investigator in seeking to initiate androgenetic development is to remove or inactivate the female pronucleus, at the same time leaving undisturbed the male pronucleus (if it is within the egg), the cytoplasm, and conditions essential for activation and first cleavage. To achieve this, especially by mechanical means, it is important that the egg be large, that the position of the egg chromatin be detectable, and that development proceed under laboratory conditions. It is, therefore, not surprising that the amphibian egg has been generally used.

G. Hertwig, in 1911, treated the eggs of *Rana fusca* with radium emanations, then fertilized them, and obtained androgenetic development for what appears to be the first time. Since then a variety of methods have been used to remove or inactivate the egg nucleus (see below). These have been applied to various European species of frogs (G. Hertwig, 1911; P. Hertwig, 1923; Dalcq, 1932) and toads (G. Hertwig, 1913; P. Hertwig, 1923), to various species of *Triton* (P. Hertwig, 1916, 1923; G. Hertwig, 1927; Curry, 1931, 1936; Baltzer, 1933; Baltzer and de Roche, 1936; Hadorn, 1934) and to one American species, *Triturus viridescens* (Kaylor, 1937).

None of these experiments has produced an adult haploid. In general, with androgenetic haploids as with haploids produced by parthenogenesis, gynogenesis and merogany, development ceases after a few days or in some cases a few weeks, is always abnormal, and where it continues to the larval stages produces an animal which is inactive and edematous.

Despite their abnormalities, these haploids offer numerous possibilities for the study of nucleo-cytoplasmic relationships. Indeed, the abnormalities in themselves are not without interest, for an experi-

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mental demonstration of their cause should throw considerable light on the problems of differentiation. To be most serviceable as an experimental material, it seems essential that the haploids and the methods by which they are produced should possess certain positive characteristics. Their development should be fairly normal and continue to an advanced stage of differentiation; the peculiarities of haploid development should be uniformly displayed by all animals; the haploid nuclear condition should remain unchanged; and the operative technique should be simple, effective, and capable of producing relatively large numbers. Haploids produced from eggs of various species of amphibia and by a variety of methods have satisfied these criteria to varying degrees, in no case perfectly. In view of this fact it is important to experiment further with new materials and methods.

The report which follows presents the results of such experiments. An effective technique for the removal of the egg chromatin from the egg of *Rana pipiens* is described; the development which results from these operated eggs is described and compared with the normal diploid; it is shown that the great majority of these animals develop as haploids; and certain cytological observations are presented which are of possible importance in explaining the abnormalities of haploid development.

I should like to express my sincere gratitude to Professor Leigh Hoadley for his aid and advice during early investigations of this material. I am also indebted to Professor G. Fankhauser for valued suggestions in more recent studies.

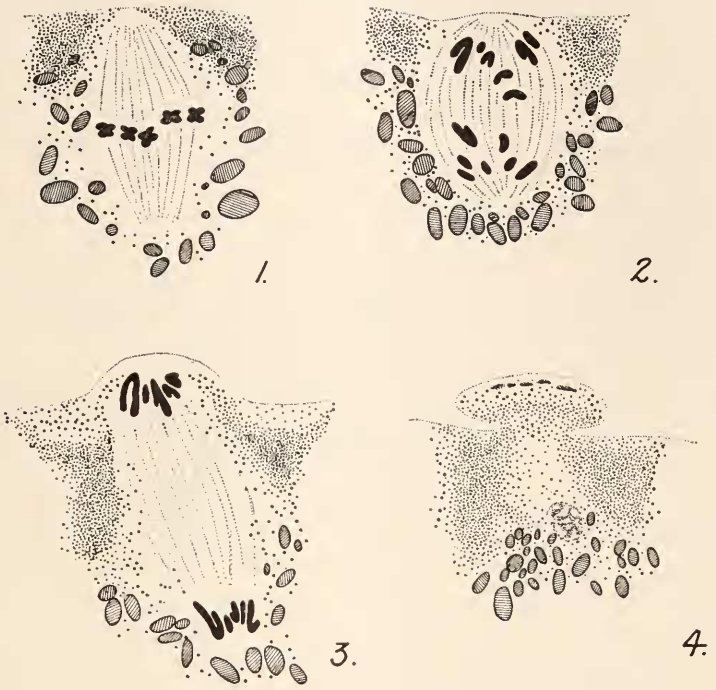
## Materials and Methods

The eggs of the frog, *Rana pipiens*, secured from the state of Vermont were used in these experiments. Ovulation was induced by injecting water extracts of the anterior lobe of the frog pituitary following in general the method described by Rugh (1934). Such eggs when inseminated usually give a high percentage of fertilization and since the development which follows is perfectly normal there is little reason for considering the eggs so obtained as inadequate for experimental purposes.

The operation, which results in the removal of the maternal chromatin, is simple and effective. Since it is in part original to these investigations and since its successful application depends on an understanding of events taking place within the egg, a rather complete description follows.

At the time of insemination the egg of *R. pipiens* has undergone the first maturation division and the second division is in metaphase

awaiting the entrance of the sperm before continuing in the production of the second polar body and the female pronucleus. Sections through the egg in this stage of maturation reveal the relation of the spindle to the egg surface. (Fig. 1). It is to be noted that it lies close to the surface and is almost completely covered over by pigment granules. As the second maturation division proceeds this relationship is altered.




FIGS. 1–4. Semi-diagrammatic representations of four stages in second polar body formation of *R. pipiens* eggs. Drawings were made with camera lucida and give exact distribution of pigment granules, yolk platelets and chromosomes, only part of which are shown. Selected from considerable material sectioned at 10$\mu$. (Eggs inseminated and kept at 12° C.) 1125 ×.

Fig. 1. Division spindle as in egg at time of insemination.

Fig. 2. Anaphase of maturation division. Stage at which spindle can be seen from exterior of egg as small black dot. Egg fixed 35 minutes after insemination.

Fig. 3. Early telophase. Egg fixed 50 minutes after insemination.

Fig. 4. Polar body just forming. Egg fixed 56 minutes after insemination.

Between 20 and 35 minutes after insemination the metaphase gives way to anaphase and the pigment granules directly over the division figure become widely dispersed (Fig. 2). If, at this time, the egg is observed from the exterior under strong illumination and a magnification of more than 25 or 30 diameters the location of the maturation spindle can be detected as a small black dot. This appearance is

doubtless due to the absence of light-reflecting pigment granules over the spindle (Figs. 2 and 3). Many of these so-called black dots have been watched and in all cases they have been observed to disappear gradually (between 35 and 45 minutes after insemination) and to be replaced by the small second polar body (Figs. 3 and 4).

The removal of the egg chromatin is accomplished by means of a glass needle possessing a very fine but rigid point. While the location of the maturation spindle is apparent the point of the needle is inserted through the jelly capsule and into the cortex of the egg to one side of and diagonally beneath the spindle (Fig. 5). A slight upward motion of the needle then produces a small exovate which contains the spindle and consequently all of the maternal chromosomes (Fig. 6). When

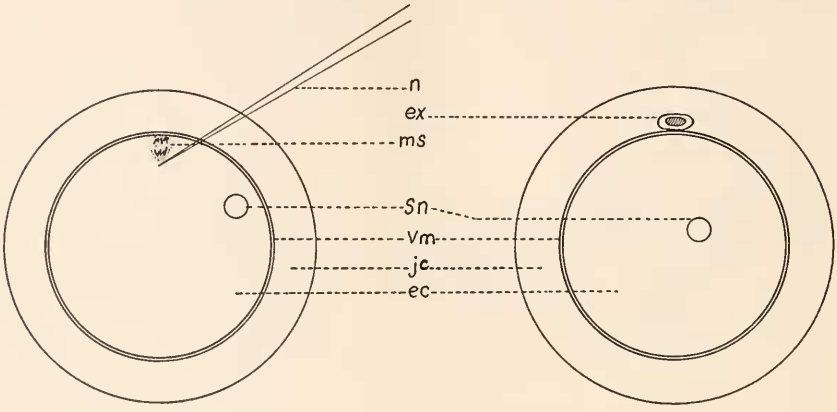



Fig. 5. Diagram of operative procedure. *n*, needle; *ms*, maturation spindle; *sn*, sperm nucleus; *vm*,vitelline membrane; *jc*, jelly capsule; *ec*, egg cytoplasm.

Fig. 6. Diagram of egg and exovate after operation. *ex*, exovate; others as in Fig. 5.

the operation is performed slowly and carefully the small pellucid spindle can occasionally be seen in the yolky cytoplasm which comes out with the needle. Thus the egg is left otherwise intact with only the male chromatin present to influence the development which follows. The exovate which forms outside the vitelline membrane is soon completely detached from the egg and generally no mark remains on the embryo to mark the place of exovate origin and former attachment.

The usefulness and value of such an operation are in part determined by the ease with which it can be executed and therefore the

number of eggs which can be treated in a short length of time. Within the 10 to 15 minutes during which the maturation spindles on a group of eggs are apparent it is possible to operate on 25 or 30 eggs and exercise considerable care in so doing. If the eggs are inseminated in small quantities and at 15-minute intervals this number can be increased several times and sufficient material is made available for quantitative studies of a physiological as well as morphological character.

The loss of the small amount of egg cytoplasm which forms the exovate appears to have no harmful effect upon later development. Evidence for this statement is drawn from the following sources: (*a*) Experiments have been performed in which small exovates were produced on eggs in the immediate vicinity of, but not including, the maturation spindle. These developed normally as far as could be observed from external appearances and certainly displayed none of the abnormalities characteristic of haploid embryos. (*b*) Occasionally a normal appearing embryo arises from an operated egg (possibly as result of unsuccessful operation). In two cases these have been allowed to develop and have ultimately metamorphosed without showing any notable deficiencies. Therefore, it seems justifiable to conclude that the abnormal characteristics of the animals which result from these operated eggs are due to an altered nucleus rather than to an altered cytoplasm.

Various other methods have been applied to amphibian eggs to bring about androgenesis. The egg chromatin has been rendered inactive by radium emanations and x-rays (G. Hertwig, 1911, 1913, 1927; P. Hertwig, 1916, 1923; Dalcq, 1929, 1932), it has been removed by pricking the egg of *R. esculenta* with heated and unheated needles (Dalcq, 1932), and it has been destroyed with a needle and then withdrawn by a micropipette (Curry, 1931, 1936; Baltzer, 1933; Baltzer and deRoche, 1936; Hadorn, 1934; Kaylor, 1937).

A comparative evaluation of these various methods should be made only by one who has tried them all. Furthermore, for different eggs, different operations may be required. For example, with the egg of *Triturus viridescens* it is necessary to use a micropipette to remove the egg chromatin for an exovate is not formed by merely pricking the egg. Therefore, whatever may be the merits or drawbacks of these various methods, it is necessary in any evaluation to consider them in conjunction with the egg to which they are applied.

Further comment should be given to Dalcq's method of pricking the egg with heated and unheated needles. It is similar to the technique applied in these experiments to the egg of *R. pipiens* but from his description it does not appear that he observed the exact location of

the maturation spindle. Instead, he pricked the egg in the lighter region in the centre of the animal pole where the maturation figure is generally, but not always, located. That he did not always remove the egg chromatin, as he himself suggests, is further indicated by the presence of 5 diploid embryos in a group of 22 which developed from operated eggs.

In all experiments to be reported, experimental animals and controls were from the same female, were inseminated simultaneously, were kept under identical conditions of temperature (generally constant to $\pm$ 0.05° C.), volume of water per animal, water change, etc. For fixing, a corrosive sublimate, acetic, formalin mixture was generally used. This has been found to be especially valuable for the younger, yolky stages for it has little hardening effect. Harris haematoxylin has been found most serviceable as a general stain. With it the nuclei stain a deep blue and the yolk granules remain a purple, thus permitting some degree of differentiation.

## Observations

### *The Development of Androgenetic Embryos*

The description of androgenetic development which follows is taken from observations on several groups of experimental animals. Developmental rates and illustrations (Figs. 7–22), however, refer to one particular group (Exp. 38–1) numbering 52 experimental animals which were raised at 19.4° C. From this group and one other, experimental animals were selected and fixed at 24-hour intervals as recorded in Tables II and III. Controls were simultaneously preserved. In this way material was provided for an examination of internal as well as external morphogenesis. While some variation is shown among the members of a single group, especially in the older stages, there is a majority which show the general features described below.

Observations were normally begun at the time of first cleavage. This may take place anywhere between 2 and 3 hours after insemination depending on the temperature at which the eggs have been inseminated. It is customary for between 90 per cent and 100 per cent of the operated eggs to divide normally and to do so simultaneously with the control eggs (Table I). This behavior, while typical for these androgenetic frog eggs, is not typical for all amphibian eggs. For example, Kaylor (1937) reports that a considerable proportion of his androgenetic *Triturus viridescens* eggs cleaved abnormally or failed to cleave at all and that 90 per cent of those for which cleavage records were available showed a significant delay in the appearance of the first cleavage furrow.

Blastula development of androgenetic *R. pipiens* embryos is quite normal. As the time for gastrulation approaches slight indentations occasionally appear in the animal hemisphere of the blastula. Since these later disappear, do not occur in all of the experimental embryos, and have been noted in the controls, they are not considered a typical abnormality of androgenetic development. Comparative examination of androgenetic and control late blastulae reveals noticeably smaller cells in the former. This observation made from the outside has been verified from sections. Though cell counts have not as yet been made, it seems probable that there are more cells in the androgenetic blastulae and that they have resulted from a more rapid rate of cell division.

Gastrulation begins approximately one hour earlier in the controls than in the experimental animals. This constitutes the first clear

TABLE I

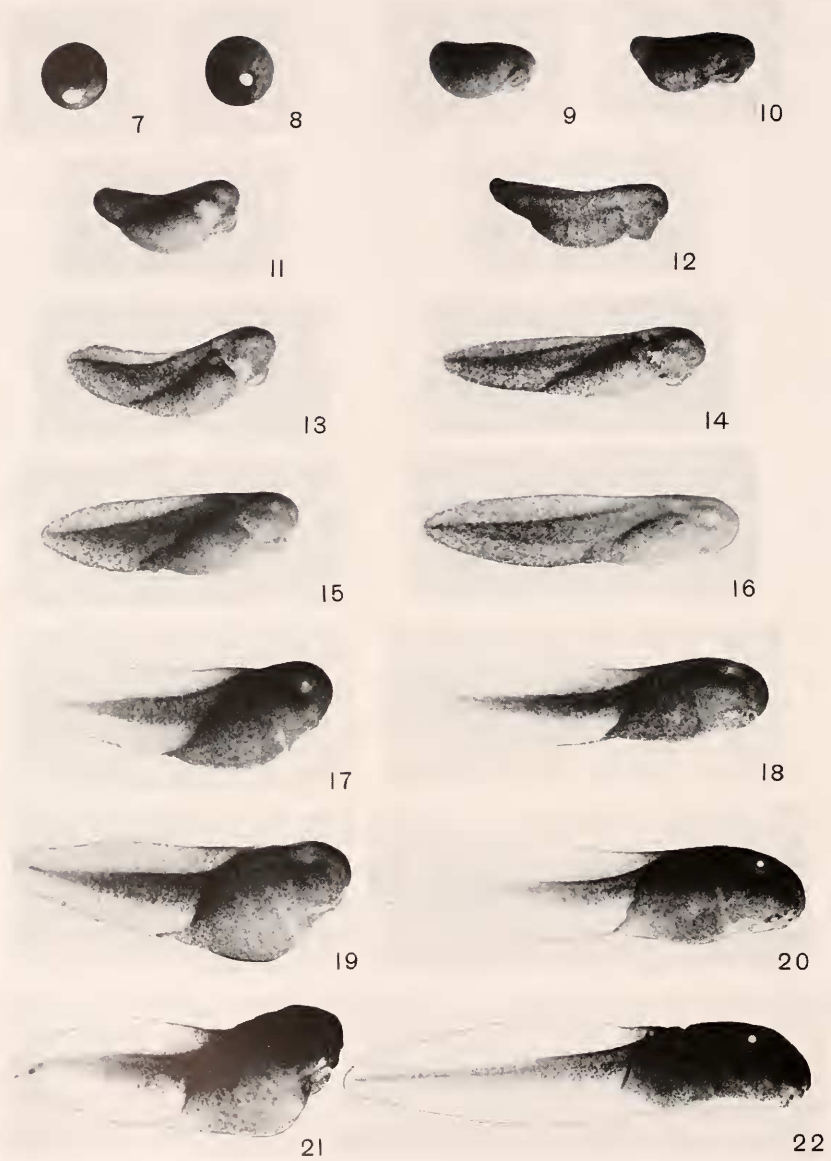
Record of first cleavage in several lots of operated eggs.

| Exp. Number | Number of Eggs Operated | Number that Cleaved | Percentage Cleavage |
|---|---|---|---|
| 36–5 | 38 | 38 | 100 |
| 36–10 | 179 | 179 | 100 |
| 36–13 | 278 | 255 | 92 |
| 37–2 | 38 | 38 | 100 |
| 37–4 | 70 | 69 | 98 |
| 38–1 | 52 | 52 | 100 |

Note: Experiments 36–10, 37–2, 37–4, and 38–1, provided the data upon which this report is based.

indication of a retardation of differentiation. This delay is a distinct characteristic of amphibian androgenetic development and has been reported by other investigators for a variety of species. It is more clearly indicated at the end of 24 hours by a difference in the size of the crescentic blastopore (smaller in the androgenetic embryos). In the great majority of the experimental animals gastrulation proceeds normally and by the end of 48 hours the yolk plug stage is reached (Figs. 7 and 8). The larger yolk plug of the androgenetic embryo provides evidence of retarded development.

During the formation of the neural tube on the third day it becomes clearly evident that androgenetic development is not simply normal development, slightly delayed, but is abnormal as well as delayed. For instance, the neural plate of the experimental animal remains approximately one-third shorter than the neural plate of the control; the neural folds stand up less prominently from the body of




FIGS. 7–22. Photographs of typical androgenetic embryos and normal diploid controls from a group of operated eggs (Exp. 38–1) raised at 19.4° C. Figs. 7, 9, 11, 13, 15, 17, 19, 21 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old androgenetic haploids. Figs. 8, 10, 12, 14, 16, 18, 20, and 22 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old controls. Photographs are of fixed animals. *ca* 5×.

the embryo, and the neural groove is more shallow. There are probably indiscernible abnormalities in gastrulation which contribute to the above and in turn to the more pronounced departures from the normal shown by the older animals. Closure of the neural folds is completed between 2 and 3 hours later than in the controls which indicates an increasing delay in differentiation.

The 3-day-old experimental animal depicts abnormal as well as delayed differentiation (Figs. 9 and 10). The tail-bud is shorter, the abdomen remains abnormally large and round, and the head is smaller and apparently less differentiated. From the third day on development is characteristic of the androgenetic embryos only, and exact stages for stage comparisons with the controls are no longer possible.

The typical 4-day-old experimental animal is smaller than the control, shows a pronounced bend in the back, a shorter and round abdomen, and a head which does not show the normal downward bend or cranial flexure (Figs. 11 and 12). The first indications of gill filaments which appear at this time in the control do not appear in the androgenetic animals until almost a day later.

Certain of these abnormalities persist on the fifth day and are clearly shown in Fig. 13. The 5-day-old control possesses a pulsating heart and a complete gill circulation whereas the experimental animals do not clearly show these features until the end of the sixth day.

In the typical 6-day-old experimental animal (Fig. 15) the back has straightened but in total length the animal is still considerably shorter than the control. It is of interest to note that the head of this animal (Fig. 15) more closely resembles that of the 5-day-old control (Fig. 14) than it does the 6-day-old (Fig. 16). But even in this similarity there are discrepancies as indicated by the position of the olfactory pit. Generally more than one-half of the androgenetic embryos of this age show a pulsating heart and of these fully one-third can be expected to have a fairly normal gill circulation.

It is typical for a few of the 7-day-old animals to become edematous and with each day thereafter the number of edematous animals increases. This condition may become so extreme that not only the body cavity but also the tissue spaces in the head become filled with fluids (Fig. 21). When this extreme is reached death generally ensues. Therefore, if the animals are to be saved, fixing agents are applied. In the group of animals from which this description is illustrated most of the animals were fixed on the ninth and tenth days (Table II).

During the eighth day the operculum grows over whatever gill filaments the animal may happen to have. This operculum development is outstanding in that it takes place at the same time and rate

as in the controls whereas other organs may be more than 24 hours delayed.

TABLE II

A record of fixation and examination for chromosome numbers of animals which developed from 52 operated eggs. (Temp. 19.4° C.)

| Age at Time of Fixation | Number Fixed | Classification |
|---|---|---|
| 1 day | 3 | 3 haploids |
| 2 days | 4 | 2 haploids<br>1 normal diploid<br>1 abnormal diploid |
| 3 days | 5 | 3 typical haploids<br>2 atypical haploids |
| 4 days | 2 | 2 typical haploids |
| 5 days | 1 | 1 haploid |
| 6 days | 3 | 1 typical haploid<br>2 atypical haploids |
| 7 days | 2 | 2 typical haploids |
| 8 days | 1 | 1 haploid |
| 9 days | 11 | 11 edematous haploids, 5 of which show a few diploid nuclei |
| 10 days | 8 | 8 edematous haploids, 7 of which show a few diploid nuclei |
| 11 days | 3 | 3 edematous haploids, 2 of which show a few diploid nuclei |
| 12 days | 6 | 4 edematous haploids showing a few diploid nuclei<br>1 edematous haploid with several diploid nuclei<br>1 diploid-triploid, developed more successfully than the typical haploid |
| 15 days | 1 | 1 haploid-diploid, haploid on one side, diploid on other side. |
| 22 days | 2 | 1 pure diploid of normal structure<br>1 triploid, appearance of normal diploid |

Summary: 46 haploids, 3 diploids, 1 triploid, 1 haploid-diploid, 1 diploid-triploid; 89 per cent of population haploids.

After the eighth day there is slight change in the gross appearance of the experimental animals except that the majority become increasingly edematous (Figs. 19 and 21). Differentiation of some

parts continues but a discussion of such differentiation is not essential to this general description. It should be mentioned, however, that in those cases where a circulation is established, at least for a short time, differentiation is more successful and the animal lives over a greater number of days.

The behavior of these animals can scarcely be called normal. Most of the time they are rather inactive and lie on their sides on the

TABLE III

A record of fixation and examination for chromosome numbers of animals which developed from 38 operated eggs. (Temp. 20.1° C.)

| Age at Time of Fixation | Number Fixed | Classification |
|---|---|---|
| 2 days | 2 | 2 typical haploids |
| 3 days | 1 | 1 typical haploid |
| 4 days | 2 | 1 typical haploid<br>1 slightly atypical haploid |
| 5 days | 2 | 2 typical haploids |
| 6 days | 1 | 1 typical haploid |
| 7 days | 10 | 1 typical haploid<br>8 edematous haploids<br>1 very atypical haploid |
| 8 days | 4 | 4 edematous haploids |
| 9 days | 11 | 10 edematous haploids, 5 of which show a few diploid nuclei<br>1 accidentally destroyed had developed as haploid |
| 10 days | 5 | 1 edematous haploid<br>2 edematous haploids showing a few diploid nuclei<br>1 haploid-tetraploid (Fig. 32)<br>1 died before fixation, had developed as haploid |

Summary: 37 haploids, 1 haploid-tetraploid; 97 per cent of population haploids.

bottom of the container. When sufficiently stimulated, however, they will respond by swimming about in undirected circles.

*Chromosome Numbers and Nuclear and Cell Size*

That the embryos which develop from operated eggs are haploids has been indicated, not only by the rather certain removal of the second polar spindle, but also by the abnormalities which they show. For further evidence, however, a cytological examination was made of some part or parts of each animal of two different groups of operated

eggs. For the younger animals this evidence was obtained from sections; for the older animals, from tail tips clipped from fixed specimens and made into whole mounts. In the case of each animal one or more metaphase plates were examined in detail to establish the chromosome number, and, in addition to this, a record was kept of the total number of division figures which could be identified as haploid or otherwise by brief examination only. In general, the quality of the preparations permitted the examination of 25 or more (in some cases many more) mitotic figures. The results of these studies are summarized in Tables II and III and additional evidence is shown in Figs. 23 to 27.

It is clearly shown that the vast majority of these operated eggs developed as haploids. For the exceptions there is at the present time no definite explanation. There always remains the possibility that they resulted from unsuccessful operations whereby the egg chromatin remained within the egg. But even if this is the explanation, the results indicate that at its worst the method is about 90 per cent effective. The animals which did not develop as haploids were easily detected for they showed either the characteristics of normal diploids or other characteristics not typical for haploids.

It is of interest to compare these results with those reported by Dalcq (1932) for androgenesis with the egg of *R. esculenta* and by Parmenter (1933) for parthenogenesis with the egg of *R. pipiens* and *R. palustris*. Out of 22 operated eggs in Dalcq's experiments 5 developed as diploids; out of 29 embryos which developed parthenogenetically Parmenter reports 10 pure diploids. These results would lead one to expect a larger number of diploids among these androgenetic *R. pipiens* embryos than have been found. In the case of Dalcq's results, however, the high percentage may be due to a poor localization of pricking and not to any marked instability of the frog haploid nucleus. But failure of operative technique could scarcely account for the large percentage of parthenogentic diploids. Several explanations, which are reviewed by Parmenter, have been suggested. It is possible that a study of very early cleavage stages will provide an explanation for this difference between the results of parthenogenetic and androgenetic experiments.

It has been noted (Tables II and III) that the tail tips of some of the older haploids show a few diploid nuclei. These were identified by their larger size and by the presence of two nucleoli (Fig. 32). Since they occur solely within the tissues of haploids which have more or less reached the end of their development, it would seem that some condition or conditions within these animals are related to their origin. But as to the mechanism of their origin, there is only slight evidence.

In a very few cases monastral divisions of haploid nuclei have been observed. The presence of scattered diploid nuclei in the older stages is not a feature confined solely to these androgenetic haploids.

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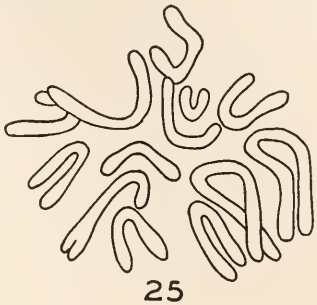

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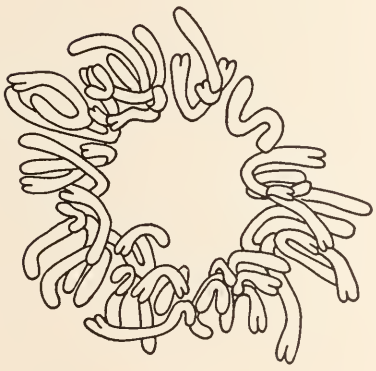

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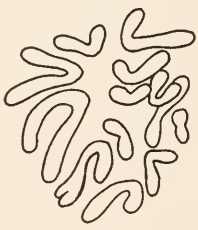

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FIGS. 23–27. Camera lucida drawings of mitotic figures. 3250 ×.

Fig. 23. Diploid metaphase from tail epithelium of 15-day-old control.

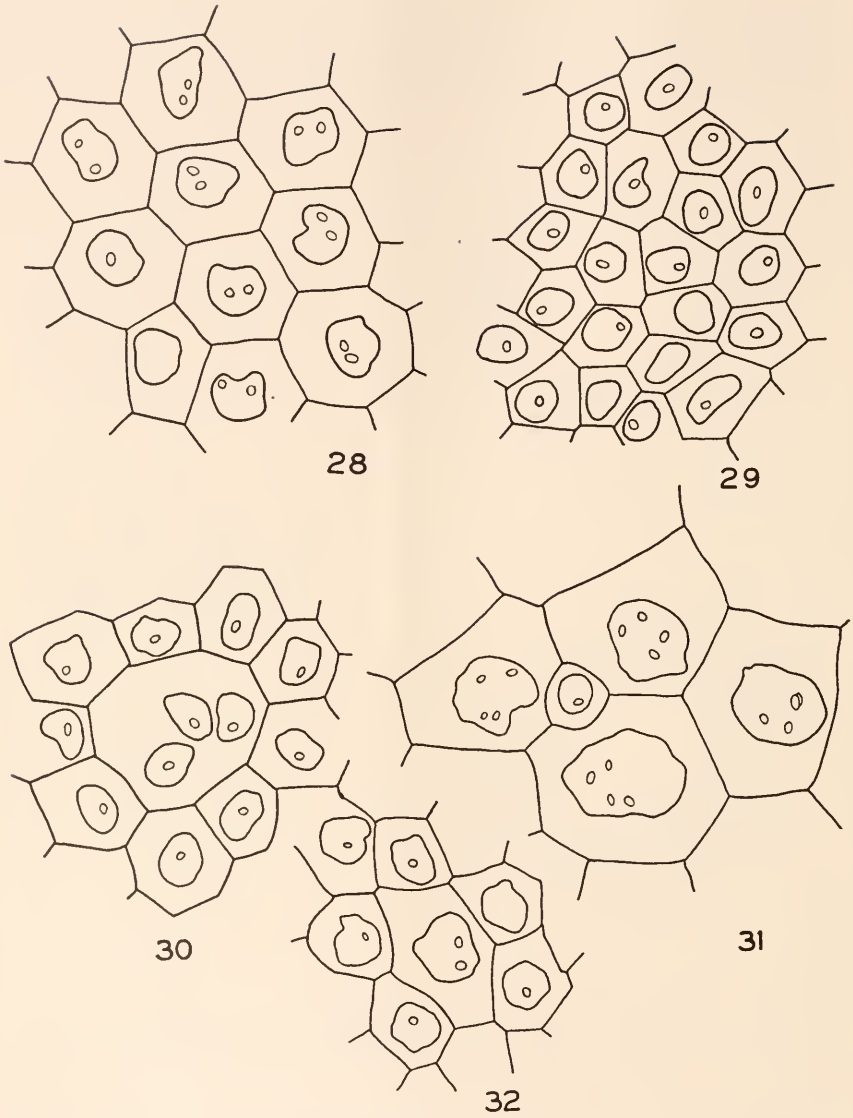
Fig. 24. Triploid metaphase from tail epithelium of 22-day-old triploid animal which developed from an operated egg (Table II). Shows 36 chromosomes (triploid 39).

Fig. 25. Haploid metaphase from 1-day-old androgenetic haploid in early stages of gastrulation.

Fig. 26. Haploid late prophase from tail epithelium of 7-day-old androgenetic haploid.

Fig. 27. Haploid metaphase from cell in tail mesoderm of 10-day-old androgenetic haploid.

Dalcq discovered the same in his preparations and Parmenter located a few diploid divisions in the tissues of some of his older animals which were otherwise predominantly haploid.




FIGS. 28–32. Camera lucida drawings of cells and nuclei from tail epithelia. 750 ×.

Fig. 28. From 9-day-old diploid control.

Fig. 29. From 9-day-old androgenetic haploid.

Fig. 30. From 9-day-old androgenetic haploid; shows 3 haploid nuclei in one large cell.

Fig. 31. From 10-day-old androgenetic embryo showing large tetraploid nuclei and cells which predominate epithelium on one side of tail (Table III).

Fig 32. From 10-day-old androgenetic haploid showing diploid nucleus and cell among haploid nuclei and cells.

The nuclear and cell size in haploids has been repeatedly shown to be smaller than in diploids and to this rule these androgenetic frog haploids are no exceptions (Figs. 28 and 29). Observations on haploids and diploids of all ages reveal that this relationship holds whether the observed animals are one day or several days old. It has also been noted that with an increase in chromosome number to triploid and tetraploid there is a corresponding increase in nuclear and cell size (Fig. 31).

There is a tendency in these haploids for several nuclei (as many as seven have been counted) to occupy a single cell. With this increase in number of nuclei, as with an increase in chromosome number, there is a corresponding increase in cell size (Fig. 30).

### *The Extent and Uniformity of Development*

Studies of groups of androgenetic embryos involving the fixation of representative types at regular intervals do not indicate accurately the extent or uniformity which might be displayed by a total population of such animals. A simple demonstration of these qualities was obtained by allowing each member of a given population of 40 animals to proceed as far as possible in its development. These animals were kept in separate containers under uniform conditions (temperature constant at 20.1° C.). While they were ultimately killed by fixing agents, the same were not applied until the indications were very definite that life would not continue for many hours. The graph presented in Fig. 33 summarizes the data of this experiment. As can readily be seen, up until the fifth day all but two of the original animals were living. From this time until the end of the eighth day there was only a slight change. At this time, however, it was necessary to preserve a large number of them because of their extreme edema. After this pronounced drop the decline is more gradual until the eleventh day after which only one animal remained alive. This one continued to live for several weeks, but, as was expected, it proved to be part haploid and part diploid. The other 39 animals were considered as haploids on the basis of the development which they displayed.

Since these animals were killed by artificial means it seemed advisable to examine some data from earlier groups of androgenetic embryos which had been raised at temperatures averaging 20° C. and in which death was caused by natural agents rather than fixing agents. The data are summarized in Fig. 34 and it is clearly evident that there was a sharp increase in the mortality rate after eight days just as depicted in Fig. 33. Hence the first graph (Fig. 33) can be considered as a correct representation of the survival value of a

population of androgenetic *R. pipiens* haploids raised at a temperature of 20° C.

It should not be concluded from these results that androgenetic frog embryos are incapable of further development than that expressed by a 10 or 12-day-old animal raised at 20° C. It is certainly true that the vast majority never go beyond this stage, but the occasional animal will continue longer and while showing abnormalities and a slower rate of growth, it will nevertheless take food and live over several weeks or months. Two animals of the group described in Fig. 34 lived for five weeks and another, which developed from an

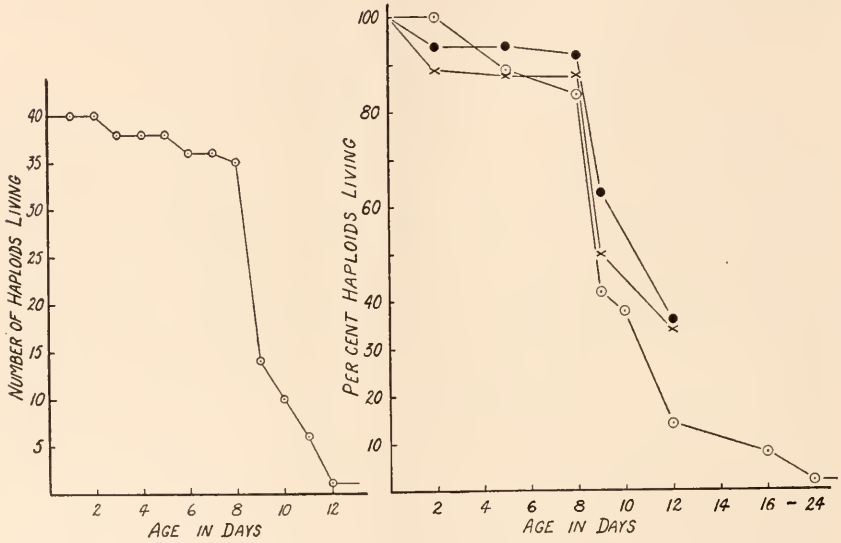



FIG. 33. Graph depicting survival of population of 40 androgenetic haploids which were fixed when it was judged that they could not survive many hours.

FIG. 34. Graph depicting survival of 3 different populations of androgenetic haploids which were allowed to die of natural causes.

operated egg in more recent experiments, lived for sixteen weeks. It developed into a sizeable tadpole with small hind limbs. Cytological examination of the tail epidermis has revealed that it was predominantly haploid.

The uniformity of a group of androgenetic embryos cannot, unfortunately, be measured by any known unit but must be left entirely to the judgment of the investigator. The fact that the majority of the animals live for eight days, suggests that early development is quite normal and uniform from animal to animal. If, on the other hand, a few animals had died each day and in all stages from cleavage to tadpole, the development could be referred to as un-uniform. The

individual animals of the group, the survival of which is described in Fig. 33, were examined every day throughout the duration of the experiment and by means of this examination were compared with one another and with one of the group selected as type. From this study the uniformity can be described as follows: until the end of the third day it was practically perfect, from the third to the fifth day it was fair and from the fifth day on it was rather poor, with differences becoming more pronounced. In other words, as the complexity of structure increased the uniformity of the population decreased.

It is difficult to compare the success (extent and uniformity) of androgenetic development displayed by these *R. pipiens* with the same development of other species. Investigators have used ages rather than stages to describe their results and in using such a unit as days-development, temperature variations become important. Among species of frogs, the androgenetic development described by G. Hertwig (1911) for *R. fusca* and by Dalcq (1932) for *R. esculenta* is no more successful than that reported here for *R. pipiens*. In fact, as far as uniformity is concerned, the results with *R. pipiens* seem to be better. This may be due to the method of operation rather than the species of egg. It has been stated that toad haploids develop better than haploids from the larger frog eggs and that *Triton* haploids develop better than the anurans (P. Hertwig, 1923). A comparative study of amphibian haploidy made at the present time might produce cause to qualify this statement. Until the haploid development of a greater variety of amphibian eggs has been studied it will be impossible to determine whether it is the species of egg, the egg size, the method of initiating haploid development or some combination of these or other factors that makes for greater success in some cases than in others.

*Internal Morphology and Development*

The typical experimental animals fixed at various ages as recorded in Tables II and III have been sectioned. The description which follows is based on an examination of these sections.

Observations on internal morphogenesis support those on external in showing that development is delayed and abnormal. These facts can be illustrated by an examination of eye development in 3-, 4-, and 5-day-old haploids and controls (Figs. 35–40). In the 3-day-old diploid (Fig. 35) the optic vesicles have extended to the head ectoderm and are in a position to induce lens formation. In the haploid (Fig. 36) the vesicles are smaller, have scarcely reached the head ectoderm and therefore show delayed development. By the end of four days, the control (Fig. 37) shows a well-formed optic cup and lens whereas the haploid (Fig. 38) has advanced only slightly beyond the stage repre-

sented by the 3-day-old control and shows only the beginning of lens formation. The 5-day-old haploid (Fig. 40) compared with the control of the same age (Fig. 39) shows an optic cup which is decidedly abnormal. Its dorsal half and the lens are quite similar to the same structures in the 4-day-old control (Fig. 37), but the ventro-lateral lips of the cup fail to grow out leaving a wide choroid fissure. It looks as if the optic stalk in failing to elongate had held in the ventral portion of the cup. Later development does not make up this deficiency in the optic cup, and by a continued proliferation of cells in the retinal layer the structure becomes increasingly abnormal. Only rarely is development more nearly normal. Thus it is observed that while development makes a fairly normal beginning as shown by the vesicle of the 3-day-old, the results as indicated by the 5-day-old and older stages are quite abnormal.

The following survey presents some further outstanding features of haploid internal morphology and morphogenesis as observed from sections of the older stages. They represent observations on the typical haploid.

*Nervous System.*—An examination of the anterior central nervous system reveals in the oldest haploids a poorly developed brain. In many cases the ventricles are almost entirely obliterated by a marked proliferation of cells or nuclei and a resulting thickening of the brain walls. This condition continues to the posterior end of the medulla. The spinal cord, on the other hand, displays a persisting neurocoele and in the caudal regions is a relatively normal structure. The nuclei are more numerous than in the diploid and in the sections of the older haploids they give way to a vacuolar type of picnosis. The fibre tracts are always indefinite in limitations and have nuclei scattered through them in an abnormal fashion.

The eye develops abnormally as indicated above. Lenses are absent in many cases and when present are considerably smaller than normal.

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FIGS. 35–40. Photomicrographs of sections through optic vesicles and optic cups of haploids and controls, aged 3, 4 and 5 days. 38 ×.

Fig. 35. From 3-day-old control.

Fig. 36. From 3-day-old haploid.

Fig. 37. From 4-day-old control.

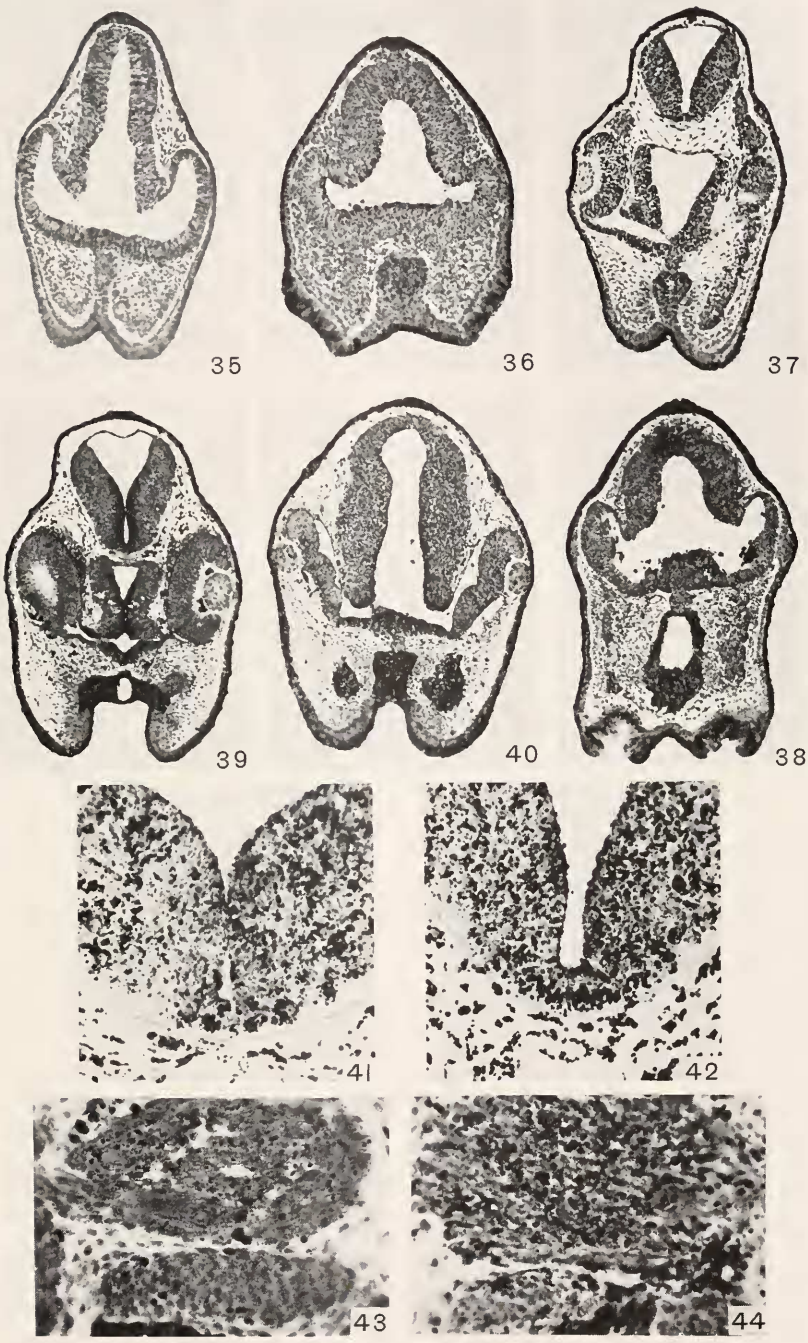
Fig. 38. From 4-day-old haploid.

Fig. 39. From 5-day-old control.

Fig. 40. From 5-day-old haploid.

FIGS. 41 AND 42. Sections through the same region of the medulla of 5-day-old control (Fig. 41) and androgenetic (Fig. 42) embryos. Yolk granules are very darkly stained inclusions. 160 ×.

FIGS. 43 AND 44. Sections through the same muscle in the pharyngeal region of 7-day-old control (Fig. 43) and androgenetic (Fig. 44) embryos. 160 ×.




FIGURES 35–40.

The otocyst, unlike the optic cup, differentiates at more nearly the normal rate, but does so abnormally. Instead of one vesicle being at first formed, several develop within the mass of cells which originally arises from the head ectoderm.

*The Notochord.*—In striking contrast with the nervous system, the notochord is among the best developed and differentiated structures in the androgenetic larvae. By the end of the third day it is well formed and displays a cross-sectional area approximately the same as that of the controls. This same relative size generally persists and when the cells become vacuolated they tend to be smaller and therefore more numerous than in the diploid. Whether or not the more successful differentiation of this structure is related to its early histogenesis is a question of some interest.

*The Pronephric Kidney.*—This appears slightly later than in the controls and shows fair development. The nephrostomes open into the body cavity and though some difficulty is encountered in tracing the course of the convoluted tubules, they appear to connect with the common duct. This latter is patent and has been traced to an open cloaca in edematous as well as in the more normal androgenetic larvae. This has its interest in that an incomplete lumen in the pronephric duct has been used to explain the edema common to these haploids (Dalcq, 1932). It is evident that such could not be the cause in all cases. The convolutions of the androgenetic kidney are less extensive than in the control kidney of the same age, which suggests a delay in elongation of the tubules. This earlier kidney is vascularized though generally to no avail as the circulation is seldom functional. Evidence for this latter fact often exists in the form of abnormal accumulations of blood cells around the tubules.

Other mesodermal derivatives such as the somites and visceral arches show fair though delayed differentiation. The somites tend to be smaller in cross-sectional area and to be underdeveloped in the thin dorsal extensions lateral to the nerve cord. The muscle cells are smaller and less compactly grouped.

*The Circulatory System.*—The circulatory system is functional in very few cases though the heart beats in many. The differentiation of the heart is considerably delayed and is generally about 24 hours behind the control in showing its first pulsations. The larger vessels can be located and traced, but the development of capillary connectives is doubtful. This latter failure is suggested by the patches of blood cells which accumulate in various regions of the body not normally associated with blood formation. Only in the occasional haploid can a good capillary circulation be located in any part of the body. The

blood cells are generally less numerous, are smaller, and contain more yolk granules. They often contain 2 or 3 nuclei after the yolk platelets have disappeared.

*The Gut.*—The gut is markedly retarded in its differentiation. This is most emphatically shown by the fact that in a 9-day-old edematous haploid the gut appears as an almost straight tube whereas in the control of the same age it is considerably coiled. The walls of this short gut are thicker and the cells are packed with yolk. The derivatives of the gut likewise differentiate rather tardily. For example, the lungs, arising from the fore-gut, are in about the same stage of development on the ninth day as they were on the seventh day in the controls. This 2-day delay in differentiation is, however, not common to the whole animal.

*The Ectoblast.*—The ectoblast in its differentiation more closely parallels the controls than any other part of the embryo. Oral suckers, olfactory pits, mouth parts and operculum all differentiate quite normally and at approximately the normal time. The ectoderm, at first wrinkled and thicker than in the controls, becomes thinner as the animal becomes edematous. Tumor-like proliferations of the ectoderm occasionally appear, and are not unlike those shown by frog embryos treated with weak solutions of 2,4-dinitrophenol (Dawson, 1938), or with high temperatures (Hoadley, 1937), or developed from over-ripe eggs (Witschi, 1930).

### *Yolk Supply*

Only a brief examination of the sections of these haploids was necessary to show that yolk disappears more slowly from the cells of the haploid than from the diploid. Since it was felt that considerable importance could be attached to this observation studies of yolk content were made along with studies of morphology. These are considered but the beginning of future studies which may throw some light on the causes of haploid deformities.

Until the haploids and controls are 4 days old (20° C.) the yolk content of the cells in all regions of the embryo is so great that microscopic comparisons are without value. In animals varying from 4 to 7 days a comparative examination of the same organs in haploids and diploids of the same age reveals a greater quantity of yolk in the cells of the haploid (Figs. 41 to 44). Within these age limits this difference holds for all tissues of the embryos though it is more apparent in some than in others. In haploids older than 7 days the yolk supply of some tissues (ectoderm of 8- and 9-day-olds) is completely exhausted while in others (the gut) it is still possible to observe a greater quantity in the cells of the haploid. It can be noted further that differentiation seems

to be more delayed in regions most richly supplied with yolk. Further observations, and if possible measurements of yolk content, are necessary before it can be stated that the cells of a haploid tissue do not approach a normal stage of differentiation until their yolk supply has been reduced to the normal extent. It is hoped that future experiments may clear up this matter and provide a basis for definite conclusions.

## Discussion

The results of these experiments indicate that the operation by which the maturation spindle is removed from the egg is successful. Approximately 90 per cent of the operated eggs develop as haploids, the haploid nucleus being that of the sperm. It has been shown that under the influence of this nucleus development proceeds for 8–10 days (20° C.) and produces a tadpole showing considerable differentiation. This is abnormal, however, and only future experiments on other eggs will indicate whether more normal haploid development is possible among the Salientia. The uniformity displayed by these populations of haploid embryos has been described as good over the first 3 days and fair from the third to the fifth days. This degree of uniformity appears to be a distinctive feature of this material for it is not clear that similar results have been previously obtained with other eggs and methods.

It is shown, therefore, that haploid embryos of suitable quality are made available in sufficient numbers for physiological studies and measurements. The abnormalities which they demonstrate occur in sufficient uniformity to make the study of their cause attractive and possibly productive. And, from another angle, they become particularly valuable as a material for hybridization experiments involving the mixing of the cytoplasm of one species with the nucleus of another.

The subject of special interest in connection with this report is the abnormal retarded development and reduced viability of these haploids, which, it is clear, must be related to the presence of only the haploid chromosome complement. Recessive genes, lethal or otherwise, unsuppressed by dominant allels would, if present in the sperm nucleus, find definite expression in these haploids. It seems hardly probable, however, that these would occur with such regularity within the male chromosomes as to produce, for example, a similar reduction in the length of the neural plate in almost every haploid in a population of 40 experimental animals. It is more logical to associate such a departure from the normal with the presence of a haploid nucleus within a quantity of cytoplasm normally associated with a diploid nucleus.

Several hypotheses have been proposed to account for these haploid abnormalities and Fankhauser (1937) finds in them a common

idea: a disturbance of the metabolism of the haploid cells. As to the nature of this disturbance there is no clear understanding, but it is presumably due to a supply of yolk and cytoplasm excessive for the haploid nucleus. There is some evidence in support of this hypothesis in the results of these investigations. It has been noted that the yolk supply disappears more slowly from the cells of the haploid than from the diploid. It has also been noted that differentiation is delayed and abnormal and that the delay appears to be more pronounced in tissues containing the greatest amount of yolk. From these observations it is not unreasonable to link excess yolk with delayed and abnormal differentiation. Additional supporting evidence comes from experiments on merogonic development. In the production of merogonic haploids the quantity of cytoplasm is more or less reduced and the normal karyoplasmic ratio tends to be restored. One such fragment of a *Triton taeneatus* egg developed through metamorphosis and constitutes the most successful case of amphibian haploidy on record (Baltzer, 1922; Fankhauser, 1938). Thus a decrease in egg cytoplasm to conform with the haploid nucleus may have permitted more normal development.

While these observations suggest a cytoplasmic influence as being responsible for the abnormalities, there is evidence which indicates that the influence in some cases arises from the nucleus. For instance, investigations of the early cleavage stages of merogonic egg fragments of *Triton palmatus* and *Triturus viridescens* have shown an unequal distribution of chromosomes (Fankhauser, 1932*c* and 1934*c*). This has been held responsible for the high mortality rate which it is customary for these merogonic embryos to show before or during gastrulation. The same explanation has been extended to the non-viable blastulae and gastrulae among *Triturus viridescens* embryos (Fankhauser and Kaylor, 1935). It is impossible, however, for any such alteration in nuclear structure to be responsible for the abnormalities of the typical frog haploid since a complete haploid complement of chromosomes has been observed in all cases studied.

Only when these studies have been extended and more is known concerning nucleo-cytoplasmic reactions whereby differentiation is brought about will it be possible to state with any certainty the conditions within the cytoplasm or nuclei of these haploids which make more normal development impossible.

## Summary

1. A technique is described by which the second maturation spindle and so all of the maternal chromatin can be removed from the egg of *R. pipiens* following its activation and penetration by the sperm.

2. The operation as applied to this egg is considered satisfactory for a large number can be treated in a short time, the maturation spindle is removed with certainty, and the slight amount of cytoplasm removed has no destructive effect on the development which follows.

3. Between 90 per cent and 100 per cent (generally 100 per cent) of the operated eggs undergo first cleavage simultaneously with the controls, and of these the majority develop for eight days, a few considerably longer.

4. The development of androgenetic haploids compared with diploid controls of the same age is abnormal and delayed. Certain features of external and internal morphogenesis are described.

5. It is shown that 90 per cent of the operated eggs can be expected to develop as haploids. This haploid nuclear condition remains unchanged until the final stages of development and then is altered only by the presence of a very few diploid nuclei.

6. As is typical for haploids, the cells and nuclei of these androgenetic embryos tend to be smaller than those of the diploid controls.

7. The development shows a high degree of uniformity from animal to animal over the first five days after which the differences become more pronounced.

8. Yolk disappearance from the cells of the haploids is notably delayed.

9. The ease of production and the success of development of *R. pipiens* androgenetic embryos seems to provide one of the best possibilities so far encountered for the study of haploid morphogenesis from eggs which normally develop as diploids.

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